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MUTATION IN BRIEF

Novel Insertions of Bruton Tyrosine Kinase in Patients with X-linked Agammaglobulinemia

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Mutations in the gene encoding Bruton tyrosine kinase (BTK) result in X-linked agammaglobulinemia (XLA), an immunodeficiency of antibody defect. By using base excision sequence scanning method (BESS) followed by direct sequencing we found in seven unrelated families with a classical XLA phenotype various mutations including six novel mutations (g.64512_64513insC, c.108_109insG, c.1700_1701insACTACAG, g.51375_51376GC>TG, g.63991_63992insGGTAGAAAAAA, c.1956_1957insCA) and a previously known silent polymorphism (c.2031C>T). Except for two mutations, the alterations affect the kinase domain. There was exceptionally high proportion of insertions in the cohort. Frameshift insertion was found altogether in five patients, three of which are on introns, one in upstream region, and one in exon 18 leading to frameshift mutation and truncation of the protein. In the intron 4 there is a substitution of two bases. Carrier detection was performed in four families. In one case the mutation was found to be *de novo*.

KEY WORDS: Bruton tyrosine kinase, *BTK*, X-linked agammaglobulinemia, XLA, BTKbase, immunodeficiency, insertion, base excision sequence scanning, BESS

INTRODUCTION

X-linked agammaglobulinemia (XLA; MIM# 300300) was the first identified primary immunodeficiency disease (PID) of antibody deficiencies (Bruton, 1952). XLA is manifested as a B cell differentiation defect. Mutations in the gene coding for Bruton tyrosine kinase (BTK) (Vetrie et al., 1993, Tsukada et al., 1993) (EMBL:HSU78027) block B cell maturation, and thus lead to decreased numbers of B-lymphocytes and an almost (complete) lack of plasma cells with consequent drastic reduction of immunoglobulins. The disease afflicts about 1/200,000 males.

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The *BTK* gene (GDB:120542) locates in to the Xq21.3-22 region in the mid-portion of the long arm of the X-chromosome (Kwan et al., 1986). Btk together with Tec, Itk, Txk and Bmx forms a distinct family, called the Tec family protein tyrosine kinases (PTKs) (for a review see Vihinen et al., 2000, Smith et al., 2001). The members of the Tec family share the same organization consisting of PH, TH, SH3, SH2, and kinase domains. Txk, however, contains in the N-terminus unique region of cysteine residues. The N-terminal pleckstrin homology (PH) domain has e.g. membrane-localizing function. The Tec homology (TH) region is unique for the Tec family. The Src homology 2 (SH2) and SH3 domains have binding functions, whereas the kinase domain is catalytic and it phosphorylates tyrosine residues of substrate proteins. Mutations in all of the domains of Btk have been shown to cause XLA (Vihinen et al., 1995, 1999, 2001). The majority of all mutations lead to truncation of the enzyme. The XLA mutation data has been collected to a database, BTKbase, available at http://bioinf.uta.fi/btkbase (Vihinen et al., 1995, 1999, 2001). In addition to mutations, it also contains clinical information for patients.

Single stranded conformational polymorphism (SSCP) followed by direct sequencing has thus far been widely used for molecular diagnosis of XLA. Here, base excision sequence scanning (BESS) method (Hawkins and Hoffman, 1997) coupled with direct sequencing was used for the mutation analysis in *BTK* gene. By using this approach, unrelated patients and four carriers were studied.

MATERIALS AND METHODS

Subjects

Seven unrelated families (7 patients), with four carriers were studied. All the patients were males. The diagnosis was based on three criteria; an absence of or severe deficiency in circulating B cells; a very low level of serum immunoglobulins especially IgG; and a history of recurrent bacterial infections. In most of the patients, infections requiring hospitalisation developed at early childhood (range 1-5 years). Due to recurrent cases of pneumonia, bronchitis, otitis media, skin infections, chronic sinusitis and asthma, immunoglobulin replacement therapy was started early. For the Finnish patients, most are currently responding favourably to IVIG infusions. The male siblings of some of the patients had familial disease history. Blood samples were drawn after informed consent was obtained from all the patients. Few days after birth the patient 5 was operated for string ileus (duodenum). During the postoperative period he developed cutane abcesses and was screened for immunodeficiency. The subject had normal granulocyte function (chemotaxis and chemiluminescense) and normal complement activity. Leucocyte count was normal as well as the lymphocyte fraction, but flow cytometry showed low B-cell fraction, which was confirmed twice during 8 months. The patient was treated with prophylactic intravenous gammaglobulin due to resistant infections.He was deceased at the age of 2 years.

PCR amplification

Blood samples from XLA patients, carriers, and controls were collected in buffer containing heparin. Genomic DNA was prepared from the whole blood using QIAamp blood midi kit (Qiagen, Germany), according to the manufacturer's instructions. The purity of the extracted DNA was tested in a 1% agarose gel.

Each of the nineteen *BTK* exons was amplified using the improved primers as described by Vo rechovský et al. (1995). PCR reactions were carried out in a 25 μl volume containing a 5'-terminal primer labelled with 5 pmoles of γ-^{32}P-dATP (3000 Ci/mmol), incorporated using T4 polynucleotide kinase. The amplification reactions had 100 ng DNA, 2 μl of BESS dNTP (Epicentre Technologies, Madison, Wisconsin), 25 pmoles of downstream primer, and 2 U DyNAzyme DNA polymerase (Finnzymes, Finland). The samples were denatured at 95°C for 4 minutes followed by 35 cycles of 95°C for 30 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 30 seconds with a final 10 minutes extension at 72°C.

BESS scanning analysis

The principile of the BESS-T&G Base Reader system (Epicentre Technologies, USA) is to incorporate a limited amount of modified G or T nucleotides to PCR products, where they serve as sites for excision after removal of the modified base. The resulting DNA ladder can be used to detect different types of mutations. The upstream primer for each exon was radioactively labelled as described above. Samples were incubated at 37°C for 30 minutes, and then inactivated by heating at 70°C for 5 minutes.

The amplified products were excised using BESS excision enzyme reaction, and subjected to 8% polyacrylamide sequencing gel. Exons revealing differences after scanning with BESS-G or BESS-T were reamplified without radiolabel. The products were resolved in a 1% agarose gel and stained with ethidium bromide. The band of interest was excised and purified using QIAquick PCR purification kit (Qiagen, Germany). The DNA sequencing was carried out in both directions using an automated ABI PRISM^{TM} 310 Genetic Analyser (PE Applied Biosystems), with the same primers as used in the PCR reactions. Detected mutations were comfirmed by comparison of the resulting sequence with the reported coding sequence (Vetrie et al., 1993).

RESULTS AND DISCUSSION

Seven different alterations in seven patients from seven unrelated families were identified and analysed using the BESS scanning method and direct sequencing (Table 1). From one of the patients was identified a previously described polymorphic alteration. The mutation and patient data were submitted to BTKbase and each case obtained a unique patient identification number (PIN) (Vihinen et al., 1998) and accession number.

Table 1. BTK Mutations in XLA Patients and Carriers

No	PIN	Accession No	Location	Protein Domain	Mutation	Amino acid change	Mother status
1	Intron 13 (6)	A0794	Intron 13	TK	g.64512_64513insC		N.D
2	Upstream (4)	A0795	Exon 2		c.108_109insG	Upstream	Not a carrier
3	@A523X538(1)	A0796	Exon 16	TK	c.1700_1701insACTACAG	p.A523fsX538	Carrier
4	Intron 4(6)	A0797	Intron 4	PH	g. 51375_51376GC>TG		Carrier
5	Intron 13(7)	A0798	Intron 13	SH2	g.63991_63992insGGTAGAAAAAA		N.D
7	@H609X649(1)	A0799	Exon 18	TK	c.1956_1957insCA	p.H609fsX649	Carrier

From the cohort several different mutations were found: five insertions, two of which lead to protein truncation, and a two base substitution in the intron 4. All the patients showed XLA phenotype. All the disease-causing mutations are novel. The silent polymorphism c.2031C>T in patient 6 has been previously described (Bradley et al., 1994; Rodriguez et al., 2001). The same alteration was found from the patient's mother.

The BESS-T&G Base Reader scanning method provides a good approach for XLA analysis in patients. Mutation was found from six out of seven families. The BESS-T system utilizes limited amount of dUTP to incorporate into the PCR product. Upon the treatment of the PCR product with the excision enzyme the uracil is removed, with cleavage occurring at the phosphodiester bonds at every thymidine site. Correspondingly, the BESS-G system incorporates modified guanidine bases. The radioactive labelling of one of the primers in both T and G systems enables mutations to be detected, subject to a sequencing gel. Mutations were localized using different parameters, which included appearance or disappearance of a band compared to a control, or change in intensity of a band compared to a control. BESS system provides certain benefits compared to other widely used mutation detection methods. It can be used to detect all kinds of mutations, polymorphisms and SNPs. The protocol is relatively simple and fast and does not require extensive optimization. The method is sensitive and can be adapted for high-throughput screening and for use with several detection methods. This is the second report of usage of BESS scanning for BTK mutation analysis. Previously, we have identified a mutation in the Btk PH domain with the technique (Okoh and Vihinen, 1999).

The clinical profiles of all the patients (Table 2) showed XLA phenotype. The patients except for subjects 2 and 5 are Finnish nationals. Some of the patients showed a positive family history of the disease and in one case the mutation was found to be *de novo*. In this family the analyses indicated that the mother, father, brother and sister all were non-carriers. Previously *de novo* mutation in oocytes or gonadal mosaicism in certain X-linked hereditary disease has been reported (Parolini et al., 1993) and recently also for XLA (Sakamoto et al., 2001). Patient 4 has been previously analysed (Okoh and Vihinen, 1999), only the carrier status was verified in here.

The identified XLA -causing mutations are novel. There was exceptionally high proportion of insertions among the analysed patients. As typical for insertions, the mutations are novel. From subject 6 was found only a previously identified polymorphism that does not alter the coded protein . This patient might have another mutation outside the range of the primers utilized or alternatively suffer from a rare autosomal recessive form of

agammaglobulinemia e.g. have mutation in μ heavy chain (Yel et al., 1996), Igα (CD79a) (Minegishi et al., 1999a), BLNK adaptor protein (Minegishi et al., 1999b), or λ5/14.1 (Minegishi et al., 1998).

Patient 2 had an upstream mutation in exon 2. This mutation in the immediate vicinity of the translation initiation site presumably affects the gene expression. Patient 1 had an insertion of a single base at intron 13 position –7 at the intron/exon border. Patient 5 had an insertion in the intron 13 at position +26. Patient 3 and 7 had frameshift insertion in exon 16 and 18, respectively, leading to a premature termination of the protein in the kinase domain and inactive enzyme.

Table 2. Clinical Data for XLA Patients[a]

No.	Age of onset (years)	Age at diag (years)	Ig level (g/l)			B cells (%)				T cells (%)			Family history
			IgG	IgM	IgA	CD19+	CD10+	CD20+	CD23+	CD3	CD4	CD8	
1	1	1	<1	<0.2	<0.2	-	-	-	-	-	-	-	none
2	1	1	<1	<0.2	<0.2	-	-	-	-	-	-	-	none
3	2	5	2.3	<0.2	<0.2	ND	ND	ND	ND	82	52	32	Positive
5	2 weeks	2 mos	2.5	0.14	<0.1	2	ND	ND	ND	69	52	25	none
7	1	4.8	<0.55	<0.02	<0.04		ND	2	-	-	-		Positive

[a]ND, not detectable

The mutation of the patient 4 is a substitution of two successive bases at position –28 and –27 in intron 4. At least some of the intron mutations could generate new alternative splice sites with altered protein sequence and structure. Protein level tests are required to prove that. These insertion mutations might disrupt or create exonic splicing enhancers or silencers, or alter pre-mRNA structure, or create new splice sites or strengthen cryptic sites.

In this report the BESS mutation scanning method was shown to identify mutations in *BTK*. Of the seven families gene disrupting mutation was found in six cases. In the remaining case a common polymorphism was identified. In this case disease-causing mutation was thought to lie outside the tested areas or to have mutation in another agammaglobulinemia-causing gene.

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